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In Vitro Chromosome Aberrations Study in Chinese Hamster Ovary (CHO) Cells

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5d. PROJECT NUMBER
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96-01

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14. ABSTRACT

In the Chinese Hamster Ovary (CHO) cell chromosome aberration assay, trifluoromethane (FE-13) induced a significant increase in clastogenic damage at concentrations in air ranging from 80.0% to 100.0% in the absence of metabolic activation. Under these same condition, 100% nitrogen also induced a significant increase in chromosomal damage to the same magnitude, suggesting the possibility that the response may reflect changes in oxygen levels rather than FE-13 specifically. In the presence of metabolic activation, FE-13 and 100.0% nitrogen induced a non significant increase in chromosomal damage. It should be noted that the level of induced damage was about the same in both the non activated and the S9 activated FE-13 treated cultures while the percentage of aberrant cells was 0.0% and 2.0% in the non activated and S9 activation control cultures, respectively, suggesting that this may account for the statistically positive increase without S9 and a statistically non significant increase with S9.

15. SUBJECT TERMS
Chromosome Aberrations, CHO Cell, Mutagenicity, FE-13, Trifluoromethane

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a. REPORT	b. ABSTRACT	c. THIS PAGE			Dr. Gunda Reddy
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Integrated Laboratory Systems

STUDY TITLE

IN VITRO CHROMOSOME ABERRATIONS STUDY IN CHINESE HAMSTER OVARY (CHO) CELLS

Project No.
ILS A073-004

Sponsor's Study Number
DAAD05-91-C-0018

Test Substance
FE-13

ILS Repository No.
96-01

Final Report Date
May 24, 1996

Sponsor
U. S. CHPPM
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QUALITY ASSURANCE INSPECTION STATEMENT

ILS Project No.: ILS A073-004
Test Substance ID: FE-13
ILS Repository No.: 96-01
Study Title: In Vitro Chromosome Aberrations Study in Chinese Hamster Ovary (CHO) Cells

This study was inspected by one or more persons of the Quality Assurance Unit of Integrated Laboratory Systems, Research Triangle Park, NC, and written status reports were submitted on the following dates:

Inspection/Audit	Date Performed	Date Reported to Study Director/Management
Study Protocol	02/20/96	02/20/96 02/26/96
Cell Harvest	04/12/96	04/12/96 04/16/96
Data Audit	05/16-17/96	05/17/96 05/24/96
Final Report Audit	05/21-22/96	05/22/96 05/24/96

Kaye Cummings
Kaye Cummings, B.S.
Quality Assurance Officer

5/24/96
Date

CERTIFICATION OF GOOD LABORATORY PRACTICES

ILS Project No.:	ILS A073-004
Test Substance ID:	FE-13
ILS Repository No.:	96-01
Study Title:	In Vitro Chromosome Aberrations Study in Chinese Hamster Ovary (CHO) Cells

This study was conducted in accordance with Good Laboratory Practice regulations as promulgated by the U. S. Environmental Protection Agency (40 CFR Part 792). Physical and chemical characterization of the test substance was conducted by the sponsor. The exception was that the purity of the positive controls was not available from the suppliers.

Raymond R. Tice, Ph.D.
Study Director

5/24/96
Date

CERTIFICATION OF CONTRACT COMPLIANCE

ILS Project No.:	ILS A073-004
Test Substance ID:	FE-13
ILS Repository No.:	96-01
Study Title:	In Vitro Chromosome Aberrations Study in Chinese Hamster Ovary (CHO) Cells

The contractor, Integrated Laboratory Systems, hereby certifies that, to the best of its knowledge and belief, the technical data delivered herewith under Contract No. DAAD05-91-C-0018 is complete, accurate, and complies with all requirements of the contract.

Raymond R. Tice

Raymond R. Tice, Ph.D.
Vice President, Research and Development

5/24/96
Date

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FINAL REPORT

1.0 Study Title:

In Vitro Chromosome Aberrations Study in Chinese Hamster Ovary (CHO) Cells

2.0 Study Identification:

ILS Project No.: A073-004
Sponsor's Study No.: DAAD05-91-C-0018

3.0 Purpose of the Study:

To evaluate the potential of the test substance to induce structural chromosome damage in cultured Chinese hamster ovary (CHO) cells in both the absence and the presence of metabolic activation.

4.0 Test Substance:

FE-13 (ILS # 96-01)

5.0 Properties of the Test substance:

5.1 Compound Characterization: The test substance was received on January 3, 1996. Determination of the test substance stability and the test substance characteristics as defined in the GLP regulations of the EPA (40 CFR Part 792) is the responsibility of the sponsor.

5.2 Storage Conditions: The test substance was stored in its shipping container at room temperature in the treatment room (see Deviation #1). Stability under these conditions has been demonstrated by the sponsor and documentation is on file with them. Chemical safety and handling information was not available; hence, the sample was regarded as hazardous.

6.0 Names of Study Director and Project Officer:

Study Director: Raymond R. Tice, Ph.D.
Project Officer: Leroy Metker

7.0 Primary Study Personnel:

Paul Andrews, M.S., Laboratory Manager
Anuradha Gurugunta Udumudi, M.S., Research Associate
Leslie Hill, B.S., Research Assistant

8.0 Names and Addresses of Sponsor and Testing Facility:

Sponsor - U.S. Army CHPPM
Bldg. E-2100
Aberdeen Proving Ground, MD 21005

Testing Facility - Integrated Laboratory Systems
800-12 Capitola Drive
Durham, NC 27713

Mailing Address - Integrated Laboratory Systems
P.O. Box 13501
Research Triangle Park, NC 27709

9.0 Study Dates:

Study Initiation Date:	March 12, 1996
Experimental Start Date:	March 12, 1996
Experimental Termination Date:	May 6, 1996
Study Completion Date:	May 24, 1996

10.0 Test System:

Exponentially growing CHO-K1 cells (obtained originally from the American Type Culture Collection, Rockville, MD) were seeded in complete medium (Ham's F-12 medium containing 10% fetal bovine serum, 2 mM L-glutamine, 100 µg streptomycin/ml and 100 units penicillin/ml) for each treatment condition at approximately 2.4 x 10^4 cells/cm^2. The flasks were incubated at 37 ± 1°C in a humidified atmosphere of 5 ± 1% CO_2 in air.

10.1 **Test System Rationale:** Among the various *in vitro* systems for detecting clastogenic activity, the CHO Chromosomal Aberration Assay is widely regarded as one of the more valuable approaches. The system has been demonstrated to be sensitive to the clastogenic activity of a variety of chemicals (1). The detection of a significantly elevated level of chromosome damage is considered an indicator of genetic damage in these cultured mammalian cells.

10.2 **Control Substances**

10.2.1 **Negative Control:** Air was the vehicle used for diluting the test substance and was included in the study as a control.

10.2.2 **Positive Controls:** Mitomycin C (MMC) at 0.10 µg/ml was used as the positive control in the nonactivated experiment. Cyclophosphamide (CP) at 20 µg/ml was used as the positive control in the S9-activated experiment. Both chemicals were dissolved in sterile distilled water prior to administration to the culture media. The purity of these two positive control chemicals was not available from the supplier.

11.0 Identification:

Using a permanent marking pen, all culturing and processing containers used in the study were uniquely identified with the ILS chemical number, dose, S9 conditions, and culture termination date. At the time the microscope slides were prepared, each sample was

coded for an unbiased cytogenetic analysis. Each slide was identified by the ILS project number, ILS project number, test substance number, coded culture number, and culture termination date.

12.0 Administration of the Test Substance:

12.1 Method of Administration and Justification: As specified by the sponsor, doses of FE-13 were freshly mixed in air on the day of treatment. Culture flasks were refed with a reduced volume of media (2.5 ml total) to facilitate diffusion of the gaseous test substance to the cells. Flasks were sealed in plastic tedlar bags, the ambient air removed by vacuum, and the test substance doses introduced via a stainless steel valve stem.

12.2 Dose Levels: Selection of the dose levels was based upon toxicity, as indicated by a decline in cell growth, or by achieving the highest possible concentration (i.e., 100.0%). If possible, the high dose was selected to give at least a $\geq$50% depression in the mitotic index and/or a >50% increase in cell cycle duration.

13.0 Type and Frequency of Tests:

13.1 Osmolality and pH Measurements: Clastogenic damage can arise through nonspecific processes related to increased osmolality or to changes in pH. The osmolality of all cultures in the toxicity test was determined at the end of the exposure period using an osmometer (Precision Systems). Also at the end of the exposure period, a check of medium pH was performed by visual inspection using the phenol red indicator present in the culture medium. Osmolality and pH measurements were to be taken for clastogenicity tests if a significant increase in osmolality (>400 mOsms) or a substantial change in pH (<6.0 or >8.0) was detected in the toxicity test cultures at doses selected for clastogenic testing.

13.2 Toxicity Test: Exponentially growing cells seeded 16-24 hours earlier were exposed to air alone and six concentrations of the test substance, from 5.0 to 100 %, for 4 hours in the absence and presence of S9. A continuous exposure in the absence of S9 was not practical due to the potential adverse effect of oxygen deprivation. A 100% nitrogen gas control was added to test the effect of oxygen deprivation for 4 hours. At the end of the exposure period, the tedlar bags were opened in a hood to vent the gas, the treatment medium removed, the cells washed, and complete medium added. Bromodeoxyuridine (BrdUrd) was then added to a final concentration of 10 µM. Following a 24-hour BrdUrd incubation period, with Colcemid (0.1 µg/ml) present for the last 2 hours, the cells were harvested by scraping with a teflon scraper. Immediately prior to Colcemid addition, the average cell density of each culture was estimated and any culture exhibiting a density of less than 10% was not harvested. Cells were harvested by scraping with a teflon scraper and then exposed to a hypotonic solution (0.075 M KCl) at 37 ± 1°C and fixed using absolute methanol, followed by 3:1 methanol: glacial acetic acid. Slides were prepared and processed for sister chromatid differentiation (stained with Hoechst 33258, exposed to blacklight, and then counterstained with Giemsa).

Duplicate cultures were evaluated for the percentage of first, second, and third plus subsequent division metaphases, based on 100 metaphase cells per culture, and for the proportion of cells at metaphase (mitotic index), based on 1000 cells per culture, and for the polyploid index (number of polyploid metaphase cells per 100 metaphase cells).

13.3 **Chromosomal Aberration Test:** Exponentially growing cells seeded 16-24 hours earlier were exposed in duplicate in tedlar bags to air alone, six concentrations of the test substance, and 100% nitrogen (as a control for oxygen deprivation) for 4 hours at 37±1°C in the absence or presence of S9. ^ continuous exposure protocol in the absence of S9 was not practical due to the potential adverse effect of oxygen deprivation. After treatment, the tedlar bags were opened in a hood to vent the gas, treatment medium removed, the cells washed with Ca and Mg free Hanks Balanced Salt Solution (HBSS) and complete medium added. The culture time from the onset of treatment to the time of termination will be approximately 1.5 average cell generations (i.e., 1.4 to 1.6 average cell generations)(3) at 37±1°C in 5 ± 1% CO_2 in air. The average generation time is generally between 12 and 16 hours in this *in vitro* culture system and will be estimated directly from the replicative index of the control cultures in the toxicity assay (4). Colcemid (final concentration of 0.1 µg/ml) was present for the final 2 hours of incubation. Cell density was visually estimated at the time of culture termination to provide an additional measure of test substance-induced toxicity and to eliminate the need for processing cultures with too few cells to analyze.

13.3.1 **Nonactivation Study:** CHO cells were exposed for 4 hours at 37 ± 1°C in 5 ± 1% CO_2 in air (2). At the end of the exposure period, the treatment medium was removed, the cells were washed with Hanks Balanced Salt Solution, refed with complete medium, and returned to the incubator for an additional 15 hours (2).

13.3.2 **S9 Activation Study:** Immediately prior to use, freshly thawed aliquots of S9 (Aroclor 1254-induced rat liver homogenates - supernatant fractions) were mixed with a cold, sterile cofactor pool in complete culture medium with reduced serum. The S9 reaction mixture was stored on ice until used. CHO cells were exposed for 4 hours at 37 ± 1°C in 5 ± 1% CO_2 in air. At the end of the exposure period, the treatment medium was removed, the cells were washed with Hanks Balanced Salt Solution, refed with complete medium, and returned to the incubator for an additional 15 hours (2).

13.3.3 **Harvesting:** Cells were harvested by scraping with a teflon scraper. The cells were collected by centrifugation, treated with warm hypotonic (0.075 M) KCl (37± 1°C), fixed first in absolute methanol, followed by methanol:glacial acetic acid (3:1 v/v), capped and stored at least overnight in a -20°C freezer. To prepare slides, the cells were collected by centrifugation and resuspended in fresh fixative. Several drops of fixed cells were dropped onto a slide, air-

dried, and stained with 4% Giemsa. Two slides were prepared from each treatment flask.

13.4 **Chromosome Aberration Analysis:** Coded slides were scored without knowledge of the dose group. In addition to the negative, positive, and nitrogen controls, the three highest treatment doses capable of providing a suitable number of metaphase cells for analysis were scored for clastogenic damage. Metaphase cells with 21 ± 2 centromeres were examined at 1000X magnification. Whenever possible, a minimum of 200 metaphase spreads from each dose group (100 metaphase cells per duplicate culture) was examined and scored for chromatid and chromosome gaps and breaks and chromatid and chromosome rearrangements, pulverized chromosome(s), pulverized cells and severely damaged cells (>10 aberrations). The number and types of aberrations found are presented for each treatment group. The percentage of structurally damaged cells in the total population of cells examined were calculated for each group. The frequency of structural aberrations per cell were calculated and reported for each group. In addition, the proportion of cells at metaphase (mitotic index) based on 1000 cells per culture, and the polyploid index, based on 100 metaphase cells per culture, was determined for each culture. In assessing the polyploid index, the proportion of endoreduplicated mitosis was determined.

14.0 Statistical Analysis:

For each metaphase evaluated, the type and number of aberrations were recorded. The mitotic index (MI), percentage of polyploidy among mitotic cells (PI), percentage of damaged cells, and cell density was determined for each culture and treatment. The statistical analysis of the chromosomal aberration data included a one-tailed Cochran-Armitage trend test analysis (5) and a one-tailed Fisher's Exact Test for a pairwise comparison of each dose group against the concurrent control. The one-tailed Cochran-Armitage trend test analysis was conducted using the Chromosome Aberration Assay Data Management and Statistical software package, version 1.0. This software package was developed by ILS for the U.S. Environmental Protection Agency in consultation with a panel of biostatisticians and experts in the field of cytogenetics (4). Fisher's Exact Test analysis was conducted using the GraphPAD Software package. The MI, PI, and cell density data were statistically analyzed by a one-way Analysis of Variance (ANOVA) followed by a two-tailed pairwise student's t comparison of each dose group against the concurrent control using the GraphPAD Software package.

15.0 Criteria for Determination of a Valid Test

15.1 **Negative Control:** The mean percentage of cells with structural aberrations in the negative control must not exceed 6%.

15.2 **Positive Control:** The positive control set of cultures must exhibit a statistically significant increase in the percentage of metaphase cells with at least one chromosomal aberration (excluding gaps).

15.3 **Test Substance:** The test substance, at least at the highest dose, should induce

at least a 50% depression in the mitotic index. However, if no cytotoxicity is observed at the limit of exposure, the assay will be considered acceptable.

16.0 Criteria for a Positive Response

The response to the test substance will be deemed positive if the following criteria are met:

A significant, dose-dependent increase in the percentage of metaphase cells containing at least one chromosomal aberration (excluding gaps) is detected. This is demonstrated by a statistically significant finding in the one-tailed trend test.

A statistically significant increase for at least one treatment dose is detected in the percentage of metaphase cells containing at least one chromosomal aberration (excluding gaps), as indicated by an appropriate pairwise comparison of each dose against the concurrent negative control treatment group.

If either, but not both, of the above conditions are met, the assay results will be evaluated by the study director and be classified as positive, equivocal, or negative depending on the nature and magnitude of the response.

If neither of the above conditions are met, the test substance is classified as negative for clastogenic activity in this *in vitro* test.

17.0 Records to be Maintained:

Data were recorded on loose work sheets adapted or prepared as necessary for the test results. All data, slides, original copies of the protocol and report, and all correspondence are archived at ILS until acceptance of the final report by the sponsor or three months after submission of the final report to the sponsor. At this time all items will be transferred to the sponsor for archiving.

18.0 Good Laboratory Practices Compliance:

The study was conducted in accordance with Good Laboratory Practice regulations as promulgated by the Environmental Protection Agency (40 CFR Part 792), except the purity of the positive controls was not available from the suppliers.

19.0 Quality Assurance:

The protocol was reviewed by the ILS QAU before final approval. A quality assurance inspection of critical phases was conducted to assure the quality and integrity of the study results. An audit of the report was conducted to determine the consistency between the reported information and the raw data.

20.0 Test Substance Disposition:

Any unused test substance and a log accounting for all test substance use will be returned to the sponsor upon completion of the contract.

21.0 Results:

21.1 Chemicals and Reagents: The chemicals and reagents used in this study were obtained from the following commercial sources:

Chemical	Source	Lot No.
Acetic acid	Fisher	956265
Bromodeoxyuridine	Sigma	34H0238
Colcemid	Irvine	931160212
Cyclophosphamide	Sigma	72H0088
Fetal bovine serum	Irvine	300341227
Giemsa	J.T. Baker	J36550
Ham's F-12	Irvine	905850826, 905860127
HBSS	Irvine	922850927
Isocitric acid	Sigma	69F-3776
Methanol	Fisher	952201, 963109
Mitomycin C	Sigma	M-0503
NADP	Sigma	114H7807
Penn/Strep	Irvine	936650428, 936650728
Potassium chloride	Sigma	15H0530
Rat liver S9	Mol Tox	0612
Sodium hydroxide	Fisher	955173-24

21.2 Toxicity Test: The test substance was tested for toxicity at 5.0, 10.0, 25.0, 50.0, 75.0, and 100.0% FE-13. A 100% nitrogen gas control was included to test the effects of oxygen deprivation on toxicity.

21.2.1 Toxicity in Nonactivation Cultures: Individual culture data are presented in Table 1 and summarized in Table 2. Over the concentrations evaluated for toxicity, the osmolality and pH in the culture media was not altered. The MI was significantly depressed only in cultures treated with 100% FE-13 (by >50%), while the cell density was not appreciably depressed at any dose. The maximal increase in average generation time (AGT) was less than one hour. The osmolality and pH in the 100.0% nitrogen control was not altered while the mitotic index and cell density were decreased slightly. Based on these results, the maximum dose of the test substance to be tested, in the absence of metabolic activation, was selected to be 100.0%.

21.2.2 Toxicity in Activation Cultures: The initial toxicity test was deemed unacceptable due to an osmolality of over 400 mOsm in the negative control cultures as well as a lack of mitosis among all almost all cultures at termination. The +S9 toxicity test was repeated over the same dose range. Individual culture data are presented in Table 3 and summarized in Table 4. Over the concentration evaluated for toxicity, the osmolality and pH in the

50% at 75.0% FE-13 but only by 28% at 100.0% FE-13. Cell density was not depressed below 10% at any dose, while the AGT remained relatively constant. The osmolality, pH, and cell density in the 100.0% nitrogen control cultures was not altered while the mitotic index was decreased slightly. Based on these results, the maximum dose of the test substance selected for testing in the presence of metabolic activation was 100.0%.

21.3 **Clastogenic Activity in Nonactivation Cultures:** CHO cells were exposed in the absence of metabolic activation to FE-13 at 50.0, 60.0, 70.0, 80.0, 90.0, and 100.0%. A 100.0% nitrogen gas control was included to test the effects of oxygen deprivation on the induction of chromosomal aberrations. In addition to the various controls, the incidence of chromosomal aberrations was assessed at 80.0, 90.0, and 100.0 % FE-13. The data are presented in Table 5 and are summarized in Table 6. FE-13 induced a significant increase in chromosomal damage, based a significant trend test ($p = 0.002$) in the percentage of damaged cells excluding gaps and by obtaining a significant increase in chromosomal damage at all three doses (80.0, 90.0, and 100.0%) evaluated for clastogenicity ($p < 0.05$). The types of induced chromosomal aberrations consisted predominantly of chromatid-type aberrations. Both the positive control, MMC at 0.10 µg/ml, and 100.0% nitrogen were clastogenic ($p < 0.01$), inducing predominantly chromatid-type chromosomal damage.

The data on MI, PI, and cell density are presented in Table 7 and summarized in Table 8. A significant depression in the MI was observed in cultures treated with FE-13 at 70.0 and 100.0% ($p < 0.05$), with a depression of >50% observed at 100.0% FE-13. The frequency of polyploid cells was significantly altered among FE-13 treated cultures ($p = 0.017$); however, no single dose was significantly different from the concurrent control culture ($p > 0.05$). Cell density among treated cultures was significantly altered ($p = 0.047$), with a significant decrease at 70.0% FE-13 only. For cultures treated with 100.0% nitrogen, the MI was depressed by almost 20%, a marginal nonsignificant response ($p = 0.051$), while cell density was not depressed and the polyploid index was not increased.

21.4 **Clastogenic Activity in S9-Activated Cultures:** CHO cells were exposed in the presence of metabolic activation to FE-13 at 50.0, 60.0, 70.0, 80.0, 90.0, and 100.0% FE-13. A 100% nitrogen gas control was included to test the effects of oxygen deprivation. The incidence of chromosomal aberrations was assessed at 80.0, 90.0, and 100.0 % FE-13. Individual culture data are presented in Table 9 and summarized in Table 10. At these concentrations, FE-13 induced a nonsignificant increase in the percentage of damaged cells, excluding gaps, as determined by a one-tailed trend test ($p = 0.091$) or by a pairwise comparison of each dose against the concurrent control ($p > 0.05$). The positive control, CP at 20.0 µg/ml, was significantly clastogenic ($p < 0.001$), while 100.0% nitrogen induced a nonsignificant increase in clastogenic damage ($p = 0.272$).

The data on MI, PI, and cell density are presented in Table 11 and summarized

in Table 12. A significant decline in MI was observed among all FE-13 treated cultures ($p < 0.05$), with the greatest depression being 40% at 100.0% FE-13. In contrast, cell density was not significantly depressed and the percentage of polyploid cells was not increased at any concentration ($p > 0.05$). For the 100.0% nitrogen exposed cultures, the MI was depressed but not significantly ($p = 0.097$), while the percentage of polyploid cells and cell density was not altered.

22.0 Conclusion:

In the CHO chromosome aberration assay, test substance FE-13 (ILS # 96-01) induced a significant increase in clastogenic damage at concentrations in air ranging from 80.0% to 100.0% in the absence of metabolic activation. Under these same condition, 100% nitrogen also induced a significant increase in chromosomal damage to the same magnitude, suggesting the possibility that the response may reflect changes in oxygen levels rather than FE-13 specifically. In the presence of metabolic activation, FE-13 and 100.0% nitrogen induced a nonsignificant increase in chromosomal damage. It should be noted that the level of induced damage was about the same in both the nonactivated and the S9 activated FE-13 treated cultures while the percentage of aberrant cells was 0.0% and 2.0% in the nonactivated and S9 activation control cultures, respectively, suggesting that this may account for the statistically positive increase without S9 and a statistically nonsignificant increase with S9.

23.0 References:

1. Preston, R.J., Au, W., Bender, M.A., Brewen, J.G., Carrano, A.V., Heddle, J.A., McFee, A.F., Wolff, S., and Wassom, J.S. (1981). Mammalian in vivo and in vitro cytogenetic assays: A report of the Gene-Tox Program. Mutat. Res. 87: 143-188.

2. Bean, C.L., Armstrong, M.J., and Galloway, S.M. (1992) Effect of sampling time on chromosome aberration yield for 7 chemicals in Chinese hamster ovary cells. Mutat. Res. 265: 31-44.

3. Margolin, B.H., Resnick, M.A., Rimpo, J.Y., Archer, P., Galloway, S.M., Bloom, A.D., and Zeiger, E. (1986). Statistical analysis for in vitro cytogenetic assays using Chinese Hamster Ovary cells. Environmental Mutagenesis 8: 183-204.

4. Kram, D., Bynum, G.D., and Tice, R.R. (1981) Bromodeoxyuridine labeling of mammalian chromosomes for the analysis of sister chromatid exchanges and cell replication kinetics. In: "Current Trends in Morphological Techniques". J.E. Johnson (ed.), CRC Press, Inc., Boca Raton, FL, pp. 147-176.

5. Chromosomal Aberration Assay Data Management and Analysis System, Version 1.0 (1990) U.S. Environmental Protection Agency, Environmental Monitoring Systems Laboratory, Las Vegas, NV.

TABLE 1. TOXICITY TEST IN CHO CELLS TREATED WITH FE-13 (ILS# 96-01) IN THE ABSENCE OF METABOLIC ACTIVATION

DOSE	SLIDE	PROLIFERATION KINETICS						MITOTIC INDEX			POLYPLOID INDEX			OSMOL.		CELL DENSITY	
(%)	#	1	2	3	RI	BU(hr)	AGT	# MITOSIS	# CELLS	%	# POLYPLOID	# MITOSIS	%	(mOSMs)	pH	%	% OF CONTROL
100.0 N_2	30	24	76	0	1.76	24	13.64	22	1000	2.2	0	100	0.0	296	N	75	76.1
	22	21	79	0	1.79	24	13.41	28	1000	2.8	1	100	1.0	300	N	94	95.4
	MEAN				1.78		13.52			2.50			0.50	298.0		84.5	85.8
0.0	27	26	62	12	1.86	24	12.90	26	1000	2.6	0	100	0.0	302	N	98	99.5
	17	12	80	8	1.96	24	12.24	40	1000	4.0	1	100	1.0	293	N	99	100.5
	MEAN				1.91		12.57			3.30			0.50	297.5		98.5	100.0
5.0	31	26	66	8	1.82	24	13.19	31	1000	3.1	1	100	1.0	299	N	95	96.4
	29	16	72	12	1.96	24	12.24	35	1000	3.5	0	100	0.0	293	N	94	95.4
	MEAN				1.89		12.72			3.30			0.50	296.0		94.5	95.9
10.0	21	29	70	1	1.72	24	13.95	38	1000	3.8	1	100	1.0	302	N	100	101.5
	24	18	78	4	1.86	24	12.90	33	1000	3.3	1	100	1.0	304	N	98	99.5
	MEAN				1.79		13.43			3.55			1.00	303.0		99.0	100.5
25.0	19	8	88	4	1.96	24	12.24	38	1000	3.8	0	100	0.0	293	N	100	101.5
	32	13	84	3	1.90	24	12.63	26	1000	2.6	1	100	1.0	296	N	98	99.5
	MEAN				1.93		12.44			3.20			0.50	294.5		99.0	100.5
50.0	23	13	87	0	1.87	24	12.83	14	1000	1.4	1	100	1.0	297	N	97	98.5
	18	22	78	0	1.78	24	13.48	31	1000	3.1	0	100	0.0	295	N	100	101.5
	MEAN				1.83		13.16			2.25			0.50	296.0		98.5	100.0
75.0	26	20	80	0	1.80	24	13.33	39	1000	3.9	1	100	1.0	298	N	97	98.5
	25	25	75	0	1.75	24	13.71	27	1000	2.7	0	100	0.0	299	N	91	92.4
	MEAN				1.78		13.52			3.30			0.50	298.5		94.0	95.4
100.0	20				Not Scorable			6	1000	0.6		Not Scorable		286	N	98	99.5
	28				Not Scorable			7	1000	0.7		Not Scorable		319	N	99	100.5
	MEAN									0.65				302.5		98.5	100.0

Abbreviations: AGT = BrdUrd Incubation time/RI, where the RI = Replicative Index = the frequency of first generation metaphase cells + 2x the frequency of second generation metaphase cells + 3x the frequency of third generation metaphase cells; N_2 = Nitrogen; Osmol. = Osmolality; N = Normal pH based on visual inspection.

TABLE 2. TOXICITY TEST SUMMARY: AVERAGE GENERATION TIME (AGT), MITOTIC INDEX (MI), POLYPLOID INDEX (PI), AND CELL DENSITY IN CHO CELLS TREATED WITH FE-13 (ILS# 96-01) IN THE ABSENCE OF METABOLIC ACTIVATION

DOSE (%)	AGT (hrs)	MI (%)	PI (%)	OSMOL. (mOSMs)	pH	CELL DENSITY (% OF CONTROL)
100.0 N_2	13.52	2.50	0.50	298.0	N	85.8
0.0	12.57	3.30	0.50	297.5	N	100.0
5.0	12.72	3.30	0.50	296.0	N	95.9
10.0	13.43	3.55	1.00	303.0	N	100.5
25.0	12.44	3.20	0.50	294.5	N	100.5
50.0	13.16	2.25	0.50	296.0	N	100.0
75.0	13.52	3.30	0.50	298.5	N	95.4
100.0	NS	0.65	NS	302.5	N	100.0

Abbreviations: AGT = BrdUrd Incubation time/RI, where the RI = Replicative Index = the frequency of first generation metaphase cells + 2x the frequency of second generation metaphase cells + 3x the frequency of third generation metaphase cells, data based on 100 metaphase cells per culture, 2 cultures per dose; MI = Mitotic Index = Percentage of metaphase cells, data based on 1000 cells per per culture, 2 cultures per dose; PI = Polyploid Index = Percentage of polyploid metaphase cells, data based on 100 metaphase cells per culture, 2 cultures per dose; NS = Not Scorable; N_2 = Nitrogen; Osmol. = Osmolality; N = Normal pH based on visual inspection.

TABLE 3. TOXICITY TEST IN CHO CELLS TREATED WITH FE-13 (ILS# 96-01) IN THE PRESENCE OF METABOLIC ACTIVATION

DOSE	SLIDE	PROLIFERATION KINETICS						MITOTIC INDEX			POLYPLOID INDEX			OSMOL.		CELL DENSITY	
(%)	#	1	2	3	RI	BU(hr)	AGT	# MITOSIS	# CELLS	%	# POLYPLOID	# MITOSIS	%	(mOSMs)	pH	%	% OF CONTROL
100.0 N2	56	20	77	3	1.83	24	13.11	52	1000	5.2	0	100	0.0	301	N	97	102.1
	59	10	89	1	1.91	24	12.57	53	1000	5.3	0	100	0.0	312	N	96	101.1
	MEAN				1.87		12.84			5.25			0.00	306.5		96.5	101.6
0.0	57	13	87	0	1.87	24	12.83	57	1000	5.7	0	100	0.0	312	N	99	100.5
	66	7	90	3	1.96	24	12.24	60	1000	6.0	0	100	0.0	308	N	98	99.5
	MEAN				1.92		12.54			5.85			0.00	310.0		98.5	100.0
5.0	55	15	81	4	1.89	24	12.70	47	1000	4.7	0	100	0.0	308	N	93	94.4
	60	17	83	0	1.83	24	13.11	39	1000	3.9	0	100	0.0	308	N	94	95.4
	MEAN				1.86		12.91			4.30			0.00	308.0		93.5	94.9
10.0	51	14	86	0	1.86	24	12.90	46	1000	4.6	0	100	0.0	299	N	95	96.4
	62	13	87	0	1.87	24	12.83	36	1000	3.6	0	100	0.0	312	N	96	97.5
	MEAN				1.87		12.87			4.10			0.00	305.5		95.5	97.0
25.0	58	8	92	0	1.92	24	12.50	28	1000	2.8	0	100	0.0	311	N	94	95.4
	65	18	82	0	1.82	24	13.19	49	1000	4.9	0	100	0.0	309	N	90	91.4
	MEAN				1.87		12.84			3.85			0.00	310.0		92.0	93.4
50.0	64	23	77	0	1.77	24	13.56	43	1000	4.3	1	100	1.0	310	N	94	95.4
	61	16	83	1	1.85	24	12.97	35	1000	3.5	0	100	0.0	299	N	91	92.4
	MEAN				1.81		13.27			3.90			0.50	304.5		92.5	93.9
75.0	54	16	83	1	1.85	24	12.97	10	1000	1.0	0	100	0.0	312	N	90	91.4
	52	20	80	0	1.80	24	13.33	39	1000	3.9	1	100	1.0	303	N	95	96.4
	MEAN				1.83		13.15			2.45			0.50	307.5		92.5	93.9
100.0	63	7	93	0	1.93	24	12.44	50	1000	5.0	0	100	0.0	301	N	90	95.7
	53	18	82	0	1.82	24	13.19	34	1000	3.4	0	100	0.0	304	N	88	93.6
	MEAN				1.88		12.81			4.20			0.00	302.5		89.0	94.7

Abbreviations: AGT = BrdUrd Incubation time/RI, where the RI = Replicative Index = the frequency of first generation metaphase cells + 2x the frequency of second generation metaphase cells + 3x the frequency of third generation metaphase cells; N_2 = Nitrogen; Osmol. = Osmolality; N = Normal pH based on visual inspection.

TABLE 4. TOXICITY TEST SUMMARY: AVERAGE GENERATION TIME (AGT), MITOTIC INDEX (MI), POLYPLOID INDEX (PI), AND CELL DENSITY IN CHO CELLS TREATED WITH FE-13 (ILS# 96-01) IN THE PRESENCE OF METABOLIC ACTIVATION

DOSE (%)	AGT (hrs)	MI (%)	PI (%)	OSMOL. (mOSMs)	pH	CELL DENSITY (% OF CONTROL)
100.0 N_2	12.84	5.25	0.00	306.5	N	101.6
0.0	12.54	5.85	0.00	310.0	N	100.0
5.0	12.91	4.30	0.00	308.0	N	94.9
10.0	12.87	4.10	0.00	305.5	N	97.0
25.0	12.84	3.85	0.00	310.0	N	93.4
50.0	13.27	3.90	0.50	304.5	N	93.9
75.0	13.15	2.45	0.50	307.5	N	93.9
100.0	12.81	4.20	0.00	302.5	N	94.7

Abbreviations: AGT = BrdUrd Incubation time/RI, where the RI = Replicative Index = the frequency of first generation metaphase cells + 2x the frequency of second generation metaphase cells + 3x the frequency of third generation metaphase cells, data based on 100 metaphase cells per culture, 2 cultures per dose; MI = Mitotic Index = Percentage of metaphase cells, data based on 1000 cells per per culture, 2 cultures per dose; PI = Polyploid Index = Percentage of polyploid metaphase cells, data based on 100 metaphase cells per culture, 2 cultures per dose; NS = Not Scorable; N_2 = Nitrogen; Osmol. = Osmolality = N = Normal pH based on visual inspection.

TABLE 5. CHROMOSOMAL ABERRATIONS IN CHO CELLS TREATED FOR FOUR HOURS WITH FE-13 (ILS# 96-01) IN THE ABSENCE OF METABOLIC ACTIVATION

DOSE	SLIDE	NO.	ABERRATION TYPES													CA/Cell		#DC		%DC	
(%)	#	CELLS	G'	G"	B'	B"	DM	TR	QR	Dic	Rg	CR	>10	PCh	PCe	+gaps	-gaps	+gaps	-gaps	+gaps	-gaps
MMC	47	100	1	4	3	4	0	5	4	0	1	0	1	0	0	0.320	0.270	15	12	15.0	12.0
	38	100	9	6	9	3	0	2	1	0	0	0	0	0	0	0.300	0.150	22	13	22.0	13.0
	TOTAL	200	10	10	12	7	0	7	5	0	1	0	1	0	0	0.310	0.210			18.50	12.50
100.0 N_2	46	100	3	0	1	2	0	0	0	0	0	0	0	0	0	0.060	0.030	5	2	5.0	2.0
	37	100	8	2	5	0	0	0	0	0	0	0	0	0	0	0.150	0.050	13	5	13.0	5.0
	TOTAL	200	11	2	6	2	0	0	0	0	0	0	0	0	0	0.105	0.040			9.00	3.50
0.0	49	100	2	0	0	0	0	0	0	0	0	0	0	0	0	0.020	0.000	2	0	2.0	0.0
	35	100	2	1	0	0	0	0	0	0	0	0	0	0	0	0.030	0.000	3	0	3.0	0.0
	TOTAL	200	4	1	0	0	0	0	0	0	0	0	0	0	0	0.025	0.000			2.50	0.00
50.0	36							Not Scored													
	39							Not Scored													
	TOTAL																				
60.0	41							Not Scored													
	33							Not Scored													
	TOTAL																				
70.0	34							Not Scored													
	48							Not Scored													
	TOTAL																				
80.0	43	100	2	2	6	2	0	0	0	0	0	0	0	0	0	0.120	0.080	10	6	10.0	6.0
	44	100	1	1	2	0	0	0	0	0	0	0	0	0	0	0.040	0.020	4	2	4.0	2.0
	TOTAL	200	3	3	8	2	0	0	0	0	0	0	0	0	0	0.080	0.050			7.00	4.00
90.0	50	100	3	0	1	1	0	0	0	0	0	0	0	0	0	0.050	0.020	5	2	5.0	2.0
	45	100	2	4	2	2	0	0	0	0	0	0	0	0	0	0.100	0.040	6	3	6.0	3.0
	TOTAL	200	5	4	3	3	0	0	0	0	0	0	0	0	0	0.075	0.030			5.50	2.50
100.0	42	100	7	2	2	0	0	0	0	0	0	0	0	0	0	0.110	0.020	11	2	11.0	2.0
	40	100	4	1	6	2	0	0	0	0	0	1	0	0	0	0.140	0.090	11	8	11.0	8.0
	TOTAL	200	11	3	8	2	0	0	0	0	0	1	0	0	0	0.125	0.055			11.00	5.00

Abbreviations: G',G" = chromatid and chromosome gaps, respectively; B',B" = chromatid and chromosome breaks, respectively; DM = double minute; TR = triradial; QR = quadriradial; Dic = dicentric; Rg - ring; CR = other complex rearrangement; >10 = more than 10 aberrations per cell; PCh = pulverized chromosome; PCe = pulverized cell; CA/cell = number of aberrations per cell (excluding PCh and PCe); #DC = number of metaphase cells with at least one aberration (PCh and PCe); MMC = Mitomycin C; N_2 = Nitrogen.

TABLE 6. SUMMARY OF CHROMOSOMAL ABERRATIONS IN CHO CELLS TREATED FOR FOUR HOURS WITH FE-13 (ILS# 96-01) IN THE ABSENCE OF METABOLIC ACTIVATION

DOSE (%)	NO. CELLS	CHROMOSOMAL ABERRATION TYPES													CA/Cell		%DC		P-Value+
		G'	G''	B'	B''	DM	TR	QR	Dic	Rg	CR	>10	PCh	PCe	+gaps	-gaps	+gaps	-gaps	
MMC	200	10	10	12	7	0	7	5	0	1	0	1	0	0	0.310	0.210	18.50	12.50	<0.001*
100.0 N_2	200	11	2	6	2	0	0	0	0	0	0	0	0	0	0.105	0.04	9.00	3.50	0.007*
0.0	200	4	1	0	0	0	0	0	0	0	0	0	0	0	0.025	0.000	2.50	0.00	
50.0							Not Scored												
60.0							Not Scored												
70.0							Not Scored												
80.0	200	3	3	8	2	0	0	0	0	0	0	0	0	0	0.080	0.050	7.00	4.00	0.004*
90.0	200	5	4	3	3	0	0	0	0	0	0	0	0	0	0.075	0.030	5.50	2.50	0.030*
100.0	200	11	3	8	2	0	0	0	0	0	1	0	0	0	0.125	0.055	11.00	5.00	<0.001*
												ONE-TAILED TREND TEST P VALUE						0.002*	

Abbreviations: G',G'' = chromatid and chromosome gaps, respectively; B',B'' = chromatid and chromosome breaks, respectively; DM = double minute; TR = triradial; QR = quadriradial; Dic = dicentric; Rg = ring; CR = other complex rearrangement; >10 = more than 10 aberrations per cell; PCh = pulverized chromosome; PCe = pulverized cell; CA/Cell = number of aberrations per cell (excluding PCh and PCe); %DC = percentage of metaphase cells with at least one (excluding PCh and PCe); MMC = Mitomycin C. P-Value+ based on pairwise comparison against concurrent control using one-tailed Fisher's exact test; N_2 = Nitrogen.

*Significantly different from control data at P <0.05.

TABLE 7. MITOTIC INDEX, POLYPLOID INDEX, AND CELL DENSITY IN CHO CULTURES EVALUATED FOR CHROMOSOMAL ABERRATIONS AFTER TREATMENT WITH FE-13 (ILS# 96-01) FOR FOUR HOURS IN THE ABSENCE OF METABOLIC ACTIVATION

DOSE (%)	SLIDE #	MITOTIC INDEX			POLYPLOID INDEX			CELL DENSITY	
		# MITOSIS	# CELLS	%	# POLYPLOID	# MITOSIS	%	%	% OF CONTROL
MMC	47	23	1000	2.3	0	100	0.0	99	102.1
	38	7	1000	0.7		Not Scorable		97	100.0
	MEAN			1.50			0.00	98.0	101.0
	SEM			0.800				1.00	1.03
100.0 N_2	46	56	1000	5.6	1	100	1.0	98	106.5
	37	61	1000	6.1	0	100	0.0	99	107.6
	MEAN			5.85			0.50	98.5	107.1
	SEM			0.250			0.500	0.5	0.54
0.0	49	71	1000	7.1	0	100	0.0	99	102.1
	35	69	1000	6.9	0	100	0.0	95	97.9
	MEAN			7.00			0.00	97.0	100.0
	SEM			0.100			0.000	2.00	2.06
50.0	36	50	1000	5.0	0	100	0.0	93	95.9
	39	44	1000	4.4	1	100	1.0	94	96.9
	MEAN			4.70			0.50	93.5	96.4
	SEM			0.300			0.500	0.50	0.52
60.0	41	49	1000	4.9	3	100	3.0	94	96.9
	33	47	1000	4.7	2	100	2.0	90	92.8
	MEAN			4.80			2.50	92.0	94.8
	SEM			0.100			0.500	2.00	2.06
70.0	34	33	1000	3.3	1	100	1.0	89	91.8
	48	50	1000	5.0	0	100	0.0	85	87.6
	MEAN			4.15			0.50	87.0	89.7
	SEM			0.850			0.500	2.00	2.06
80.0	43	52	1000	5.2	2	100	2.0	94	96.9
	44	48	1000	4.8	3	100	3.0	91	93.8
	MEAN			5.00			2.50	92.5	95.4
	SEM			0.200			0.500	1.50	1.55
90.0	50	52	1000	5.2	1	100	1.0	93	95.9
	45	47	1000	4.7	2	100	2.0	96	99.0
	MEAN			4.95			1.50	94.5	97.4
	SEM			0.250			0.500	1.5	1.55
100.0	42	37	1000	3.7	2	100	2.0	94	100.5
	40	28	1000	2.8	2	100	2.0	95	101.6
	MEAN			3.25			2.00	94.5	101.1
	SEM			0.450			0.000	0.5	0.53

N_2 = Nitrogen; MMC = Mitomycin C.

TABLE 8. SUMMARY OF MITOTIC INDEX, POLYPLOID INDEX, AND CELL DENSITY FOR CHO CELLS EVALUATED FOR CHROMOSOMAL ABERRATIONS AFTER TREATMENT WITH FE-13 (ILS# 96-01) FOR FOUR HOURS IN THE ABSENCE OF METABOLIC ACTIVATION

DOSE (%)	MITOTIC INDEX (%)			POLYPLOID INDEX (%)			CELL DENSITY (% OF CONTROL)		
	MEAN	SEM	P-VALUE+	MEAN	SEM	P-VALUE+	MEAN	SEM	P-VALUE
MMC	1.50	0.800	0.021*	0.00	-	ND	101.0	1.03	0.699
100.0 N_2	5.85	0.250	0.051	0.50	0.500	0.423	107.1	0.54	0.543
0.0	7.00	0.100		0.00	0.000		100.0	2.06	
50.0	4.70	0.300	0.056	0.50	0.500	0.491	96.4	0.52	0.253
60.0	4.80	0.100	0.061	2.50	0.500	0.053	94.8	2.06	0.151
70.0	4.15	0.850	0.038*	0.50	0.500	0.491	89.7	2.06	0.045*
80.0	5.00	0.200	0.073	2.50	0.500	0.053	95.4	1.55	0.178
90.0	4.95	0.250	0.069	0.00	1.000	0.129	97.4	1.55	0.374
100.0	3.25	0.450	0.022*	2.00	0.000	0.079	101.1	0.53	0.374
ANOVA P-VALUE	0.008*			0.017*			0.047*		

MI = Mitotic Index = Percentage of metaphase cells, data based on 1000 cells per culture, 2 cultures per dose; PI = Polyploid Index; Percentage of polyploid metaphase cells, data based on 100 metaphase cells per culture, 2 cultures per dose. CP = cyclophosphamide. P-Value+ based on two-tailed student t test against concurrent control; N_2 = Nitrogen; Not Scorable; ND = Not Determinable; *Significant at $p < 0.05$.

TABLE 9. CHROMOSOMAL ABERRATIONS IN CHO CELLS TREATED FOR FOUR HOURS WITH FE-13 (ILS# 96-01) IN THE PRESENCE OF METABOLIC ACTIVATION

DOSE	SLIDE	NO.	CHROMOSOMAL ABERRATION TYPES													CA/Cell		#DC		%DC	
(%)	#	CELLS	G'	G"	B'	B"	DM	TR	QR	Dic	Rg	CR	>10	PCh	PCe	+gaps	-gaps	+gaps	-gaps	+gaps	-gaps
CP	77	50	3	3	18	9	0	8	5	0	0	2	0	0	0	0.960	0.840	27	25	54.0	50.0
	70	50	5	8	28	9	0	7	9	0	1	2	0	0	0	1.380	1.120	32	29	64.0	58.0
	TOTAL	100	8	11	46	18	0	15	14	0	1	4	0	0	0	1.170	0.980			59.00	54.00
100.0 N_2	80	100	5	2	1	0	0	0	0	0	0	0	0	0	0	0.080	0.010	8	1	8.0	1.0
	71	100	8	2	5	1	0	0	0	0	0	0	0	0	0	0.160	0.060	13	6	13.0	6.0
	TOTAL	200	13	4	6	1	0	0	0	0	0	0	0	0	0	0.120	0.035			10.50	3.50
0.0	68	100	2	3	3	0	0	0	0	0	0	0	0	0	0	0.080	0.030	8	3	8.0	3.0
	74	100	3	1	2	0	0	0	0	0	0	0	0	0	0	0.060	0.020	5	1	5.0	1.0
	TOTAL	200	5	4	5	0	0	0	0	0	0	0	0	0	0	0.070	0.025			6.50	2.00
50.0	72							Not Scored													
	79							Not Scored													
	TOTAL																				
60.0	84							Not Scored													
	81							Not Scored													
	TOTAL																				
70.0	69							Not Scored													
	76							Not Scored													
	TOTAL																				
80.0	75	100	4	1	1	1	0	0	0	0	0	0	0	0	0	0.070	0.020	6	2	6.0	2.0
	78	100	2	1	2	0	0	0	0	0	0	0	0	0	0	0.050	0.020	3	2	3.0	2.0
	TOTAL	200	6	2	3	1	0	0	0	0	0	0	0	0	0	0.060	0.020			4.50	2.00
90.0	82	100	1	0	3	0	0	1	0	0	0	0	0	0	0	0.050	0.040	5	4	5.0	4.0
	83	100	4	2	4	3	0	0	0	0	0	0	0	0	0	0.130	0.070	10	6	10.0	6.0
	TOTAL	200	5	2	7	3	0	1	0	0	0	0	0	0	0	0.090	0.055			7.50	5.00
100.0	67	100	4	3	5	1	0	0	0	0	0	0	0	0	0	0.130	0.060	10	5	10.0	5.0
	73	100	3	2	3	0	0	0	0	0	0	0	0	0	0	0.080	0.030	8	3	8.0	3.0
	TOTAL	200	7	5	8	1	0	0	0	0	0	0	0	0	0	0.105	0.045			9.00	4.00

Abbreviations: G',G" = chromatid and chromosome gaps, respectively; B',B" = chromatid and chromosome breaks, respectively; DM = double minute; TR = triradial; QR = quadriradial; Dic = dicentric; Rg - ring; CR = other complex rearrangement; >10 = more than 10 aberrations per cell; PCh = pulverized chromosome; PCe = pulverized cell; CA/cell = number of aberrations per cell; #DC = number of metaphase cells with at least one aberration (PCh and PCe are not included in these calculations); %DC = percentage of metaphase cells with at least one aberration (PCh and PCe are not included in these calculations); CP = cyclophosphamide; N_2 = Nitrogen.

TABLE 10. SUMMARY OF CHROMOSOMAL ABERRATIONS IN CHO CELLS TREATED FOR FOUR HOURS WITH FE-13 (ILS# 96-01) IN THE PRESENCE OF METABOLIC ACTIVATION

DOSE (%)	NO. CELLS	CHROMOSOMAL ABERRATION TYPES													CA/Cell		%DC		P-Value+
		G'	G''	B'	B''	DM	TR	QR	Dic	Rg	CR	>10	PCh	PCe	+gaps	-gaps	+gaps	-gaps	
CP	100	8	11	46	18	0	15	14	0	1	4	0	0	0	1.170	0.980	59.00	54.00	<0.001
100.0 N_2	200	13	4	6	1	0	0	0	0	0	0	0	0	0	0.120	0.035	10.50	3.50	0.272
0.0	200	5	4	5	0	0	0	0	0	0	0	0	0	0	0.070	0.025	6.50	2.00	
50.0							Not Scored												
60.0							Not Scored												
70.0							Not Scored												
80.0	200	6	2	3	1	0	0	0	0	0	0	0	0	0	0.060	0.020	4.50	2.00	0.362
90.0	200	5	2	7	3	0	1	0	0	0	0	0	0	0	0.090	0.055	7.50	5.00	0.086
100.0	200	7	5	8	1	0	0	0	0	0	0	0	0	0	0.105	0.045	9.00	4.00	0.190
															ONE-TAILED TREND TEST P VALUE			0.091	

Abbreviations: G',G'' = chromatid and chromosome gaps, respectively; B',B'' = chromatid and chromosome breaks, respectively; DM = double minute; TR = triradial; QR = quadriradial; Dic = dicentric; Rg = ring; CR = other complex rearrangement; >10 = more than 10 aberrations per cell; PCh = pulverized chromosome; PCe = pulverized cell; CA/Cell = number of aberrations per cell (excluding PCh and PCe); %DC = percentage of metaphase cells with at least one (excluding PCh and PCe); CP = cyclophosphamide. P-Value+ based on pairwise comparison against concurrent control using one-tailed Fisher's exact test; N_2 = Nitrogen.

*Significantly different from control data at P <0.05.

TABLE 11. MITOTIC INDEX, POLYPLOID INDEX, AND CELL DENSITY IN CHO CULTURES EVALUATED FOR CHROMOSOMAL ABERRATIONS AFTER TREATMENT WITH FE-13 (ILS# 96-01) FOR FOUR HOURS IN THE PRESENCE OF METABOLIC ACTIVATION

DOSE (%)	SLIDE #	MITOTIC INDEX			POLYPLOID INDEX			CELL DENSITY	
		# MITOSIS	# CELLS	%	# POLYPLOID	# MITOSIS	%	%	% OF CONTROL
CP	77	5	1000	0.5		Not Scorable		98	100.5
	70	9	1000	0.9	0	100	0.0	97	99.5
	MEAN			0.70			0.0	97.5	100.0
	SEM			0.200				0.50	0.51
100.0 N_2	80	57	1000	5.7	0	100	0.0	96	99.5
	71	63	1000	6.3	0	100	0.0	97	100.5
	MEAN			6.00			0.0	96.5	100.0
	SEM			0.300			0.00	0.50	0.52
0.0	68	74	1000	7.4	0	100	0.0	97	99.5
	74	86	1000	8.6	0	100	0.0	98	100.5
	MEAN			8.00			0.0	97.5	100.0
	SEM			0.600			0.00	0.50	0.51
50.0	72	58	1000	5.8	0	100	0.0	94	96.4
	79	53	1000	5.3	0	100	0.0	87	89.2
	MEAN			5.55			0.0	90.5	92.8
	SEM			0.250			0.00	3.50	3.59
60.0	84	50	1000	5.0	0	100	0.0	98	100.5
	81	53	1000	5.3	0	100	0.0	95	97.4
	MEAN			5.15			0.0	96.5	99.0
	SEM			0.150			0.00	1.50	1.54
70.0	69	55	1000	5.5	0	100	0.0	97	99.5
	76	60	1000	6.0	0	100	0.0	94	96.4
	MEAN			5.75			0.0	95.5	97.9
	SEM			0.250			0.00	1.50	1.54
80.0	75	46	1000	4.6	0	100	0.0	98	100.5
	78	51	1000	5.1	0	100	0.0	97	99.5
	MEAN			4.85			0.0	97.5	100.0
	SEM			0.250			0.00	0.50	0.51
90.0	82	58	1000	5.8	0	100	0.0	93	95.4
	83	50	1000	5.0	0	100	0.0	94	96.4
	MEAN			5.40			0.0	93.5	95.9
	SEM			0.400			0.00	0.50	0.51
100.0	67	46	1000	4.6	0	100	0.0	97	99.5
	73	50	1000	5.0	0	100	0.0	95	97.4
	MEAN			4.80			0.0	96.0	98.5
	SEM			0.200			0.00	1.00	1.03

N_2 = Nitrogen; CP = Cyclophosphamide.

TABLE 12. SUMMARY OF MITOTIC INDEX, POLYPLOID INDEX, AND CELL DENSITY FOR CHO CELLS EVALUATED FOR CHROMOSOMAL ABERRATIONS AFTER TREATMENT WITH FE-13 (ILS# 96-01) FOR FOUR HOURS IN THE PRESENCE OF METABOLIC ACTIVATION

DOSE (%)	MITOTIC INDEX (%)			POLYPLOID INDEX (%)			CELL DENSITY (% OF CONTROL)		
	MEAN	SEM	P-VALUE+	MEAN	SEM	P-VALUE+	MEAN	SEM	P-VALUE+
CP	0.70	0.200	0.007*	0.00	ND	ND	100.0	0.51	1.000
100.0 N_2	6.00	0.300	0.097	0.00	0.000	ND	100.0	0.52	0.293
0.0	8.00	0.600		0.00	0.000		100.0	0.51	
50.0	5.55	0.250	0.035*	0.00	0.000	ND	92.8	3.59	0.093
60.0	5.15	0.150	0.026*	0.00	0.000	ND	99.0	1.54	0.706
70.0	5.75	0.250	0.041*	0.00	0.000	ND	97.9	1.54	0.476
80.0	4.85	0.250	0.022*	0.00	0.000	ND	100.0	0.51	>0.800
90.0	5.40	0.400	0.031*	0.00	0.000	ND	95.9	0.51	0.224
100.0	4.80	0.200	0.021*	0.00	0.000	ND	98.5	1.03	0.581
ANOVA P-VALUE	0.003*			ND			0.139		

MI = Mitotic Index = Percentage of metaphase cells, data based on 1000 cells per culture, 2 cultures per dose; PI = Polyploid Index; Percentage of polyploid metaphase cells, data based on 100 metaphase cells per culture, 2 cultures per dose. CP = cyclophosphamide. P-Value+ based on two-tailed student t test against concurrent control; N_2 = Nitrogen; ND = Not Determinable; *Significant at $p < 0.05$.

APPENDIX

PROTOCOL, AMENDMENTS, & DEVIATIONS

INTEGRATED LABORATORY SYSTEMS

STUDY PROTOCOL

1.0 Study Title:

In Vitro Chromosome Aberrations Study in Chinese Hamster Ovary (CHO) Cells

2.0 Study Identification:

ILS Project No.: A073-004
Sponsor's Study No.: DAAD05-91-C-0018

3.0 Purpose of the Study:

To evaluate the potential of the test substance to induce structural chromosome damage in cultured Chinese hamster ovary (CHO) cells in both the absence and the presence of metabolic activation.

4.0 Name and Address of Sponsor:

U.S. Army CHPPM
Bldg E-2100
Aberdeen Proving Ground, MD 21005

5.0 Name and Address of Testing Facility:

Shipping Address: Integrated Laboratory Systems
800-12 Capitola Drive
Durham, NC 27713

Mailing Address: Integrated Laboratory Systems
P.O. Box 13501
Research Triangle Park, NC 27709

6.0 Proposed Study Dates:

Proposed Experimental Start Date: February 26, 1996
Proposed Experimental Termination Date: May 10, 1996

7.0 Names of Study Director and Project Officer:

Study Director: Raymond R. Tice, Ph.D.
Project Officer: Leroy Metker

8.0 Test Substance:

8.1 Identification: FE-13 (ILS # 96-01)

8.2 Properties of the Test Substance:

8.2.1 Compound Characterization: Determination of test substance stability and test substance characteristics is the responsibility of the sponsor. Information on the test substance including its method of synthesis, analysis, physicochemical characteristics, and bulk stability is retained on file by the sponsor.

8.2.2 Storage Conditions: The test substance will be stored at room temperature in the ILS Chemical Repository. Stability under these conditions has been demonstrated by the sponsor and documentation is on file with them. Normal safety precautions appropriate for potential clastogens will be necessary when handling the test substance. A material safety data sheet will not be provided.

9.0 Test System:

9.1 Test System Justification: Among the various *in vitro* systems for detecting clastogenic activity, the CHO Chromosomal Aberration Assay is widely regarded as one of the more valuable approaches. The system has been demonstrated to be sensitive to the clastogenic activity of a variety of chemicals (1). The detection of a significantly elevated level of chromosome damage is considered an indicator of genetic damage in these cultured mammalian cells.

9.2 Culturing: Exponentially growing CHO-K1 cells (obtained originally from the American Type Culture Collection, Rockville, MD) are seeded in complete medium (Ham's F-12 medium containing 10% fetal bovine serum, 2 mM L-glutamine, 100 µg streptomycin/ml and 100 units penicillin/ml) for each treatment condition at approximately 2.4×10^4 cells/cm². The flasks are incubated at 37 ± 1°C in a humidified atmosphere of 5 ± 1% CO_2 in air.

10.0 Experimental Design:

10.1 Preparation of the Dosage Formulation: As specified by the Sponsor, test substance doses will be freshly mixed with air on the day of treatment.

10.2 Method of Administration and Justification: On the day of treatment, culture flasks will be refed with a reduced volume of media (2.5 ml total) to facilitate diffusion of the gaseous test substance to the cells. Culture flasks will be sealed in plastic tedlar bags, the ambient air removed by vacuum, and the test substance doses introduced via a stainless steel valve stem.

10.3 S9 Activation System: Immediately prior to use, freshly thawed aliquots of Aroclor 1254-induced rat liver homogenates (S9 fraction) (Molecular Toxicology, Rockville, MD) will be mixed with a sterile cofactor pool. The S9 reaction mixture will be stored on ice until used.

10.4 Dose Levels: Selection of the dose levels will be based on toxicity, as indicated by a decline in cell growth potential in the initial toxicity assay(s). Whenever possible, the high dose will be selected to give at least 50% toxicity (i.e., a 50% depression in mitotic index or a 50% reduction in cell density at the time of culture termination). In the event the test substance cannot be delivered at a high enough concentration to be cytotoxic, the study will be conducted with the maximum concentrations obtainable. Precipitation of the test substance in the culture medium will be permitted at the high dose only. Once the dose levels are determined, they will be added to the protocol in the form of an amendment.

10.4.1 Negative Control: The negative control will consist of cultures treated with 100 % air.

10.4.2 Positive Controls: Positive controls are included to demonstrate the adequacy of the experimental conditions to detect known clastogens. The positive controls included in the chromosome aberration assay will consist of mitomycin C, a direct acting clastogen, at 100 ng/ml, and cyclophosphamide, a clastogen requiring metabolic activation, at 20 µg/ml. Mitomycin C will be used as a positive control in the nonactivation assay, while cyclophosphamide will be used in the S9 activation assay. Both positive control substances will be dissolved in sterile distilled water and administered in a dosing volume of 50 µl per culture. Information on source, lot number, and purity (if provided by the manufacturer) for the positive control chemicals will be included in the final report.

10.5 Identification: Using a permanent marking pen, all culturing and processing containers used in the study will be uniquely identified with the ILS chemical number, S9 condition, dose, and culture termination date. At the time the microscope slides are prepared, each sample will be coded with a unique identification number for an unbiased cytogenetic analysis. Microscope slides prepared from the fixed cells will be permanently labeled with the project number, test substance number, coded culture number, and culture termination date.

10.6 Osmolality and pH Measurements: Clastogenic damage can arise through nonspecific processes related to increased osmolality or to changes in pH (2). The osmolality of all treated and control cultures used in the toxicity assay will be determined at the end of the exposure period using an osmometer (Precision Systems). Also, a check of medium pH will be performed at the end of the exposure period by inspection using the phenol red indicator present in the culture media unless precluded by an alteration in media color due to the test substance. If a substantial pH change occurs which can be detected visually, an aliquot will be removed and the pH quantitated using a pH meter. If a significant

increase in osmolality of the culture medium (>400 mOsms) or a significant change in pH (<6.0 or >8.0) is detected at doses selected for clastogenic testing, such measurements will also be taken for additional toxicity tests or the CA tests.

10.7 **Toxicity Assay:** Exponentially growing cells seeded 16-24 hours earlier will be exposed in tedlar bags to air alone and six concentrations of the test substance (5, 10, 25, 50, 75, and 100% FE-13) and 100% nitrogen (as a control for oxygen deprivation) for 4 hours at 37±1°C in the absence or presence of S9. Prior to use, freshly thawed aliquots of S9 (Aroclor 1254-induced rat liver homogenates - supernatant fractions) will be mixed with a cold, sterile cofactor pool in complete culture medium with reduced serum. The S9 reaction mixture will be stored on ice until used. A continuous exposure protocol in the absence of S9 is not practical due to the potential adverse effect of oxygen deprivation. After treatment, the tedlar bags will be opened in a hood to vent the gas, the treatment medium removed, the cells washed with Ca and Mg free Hanks Balanced Salt Solution (HBSS), and complete medium added. At this time, bromodeoxyuridine (BrdUrd) will be added to a final concentration of 10 µM and the cells cultured for 24- to 26-hours at 37±1°C in 5 ± 1% CO_2 in air. Colcemid (0.1 µg/ml) will be present for the last 2 hours of culture. Immediately prior to Colcemid addition, the average cell density of each culture will be recorded. Any culture exhibiting a density of less than 10% will not be harvested. Cells will be harvested by scraping with a teflon scraper. The collected cells will be exposed to a hypotonic solution (0.075 M) of potassium chloride at 37 ± 1°C and then fixed using absolute methanol, followed by 3:1 methanol:glacial acetic acid. The fixed cells will be stored in a -20°C freezer prior to slide preparation. Slides will be prepared and processed for sister chromatid differentiation (stained with Hoechst 33258, exposed to blacklight, and then stained with Giemsa). Duplicate cultures will be evaluated for the percentage of first, second, and third plus subsequent division metaphase cells, based on 100 metaphase cells per culture, for the proportion of cells at metaphase (mitotic index), based on 1000 cells per culture, and for the polyploid index (number of polyploid metaphase cells per 100 metaphase cells).

10.8 **Chromosomal Aberration Assay:** Exponentially growing cells seeded 16-24 hours earlier will be exposed in duplicate in tedlar bags to air alone, at least five concentrations of the test substance, and 100% nitrogen (as a control for oxygen deprivation) for 4 hours at 37±1°C in the absence or presence of S9. Prior to use, freshly thawed aliquots of S9 (Aroclor 1254-induced rat liver homogenates - supernatant fractions) will be mixed with a cold, sterile cofactor pool in complete culture medium with reduced serum. The S9 reaction mixture will be stored on ice until used. Selection of the test substance concentrations selected will be based on the findings from the toxicity assay and will be added to the protocol in the form of an amendment. A continuous exposure protocol in the absence of S9 is not practical due to the potential adverse effect of oxygen deprivation. After treatment, the tedlar bags will opened in a hood to vent the gas, treatment medium will be removed, the cells washed with Ca and Mg free Hanks Balanced Salt Solution (HBSS) and complete medium added. The culture time from the onset of treatment to the time of termination will be approximately 1.5 average cell

generations (i.e., 1.4 to 1.6 average cell generations)(3) at 37±1°C in 5 ± 1% CO_2 in air. The average generation time is generally between 12 and 16 hours in this *in vitro* culture system and will be estimated directly from the replicative index of the control cultures in the toxicity assay (4). Colcemid (final concentration of 0.1 µg/ml) will be present for the final 2 hours of incubation.

10.8.1 **Harvesting:** Immediately prior to Colcemid addition, the average cell density of each culture will be recorded. Any culture exhibiting a density of less than 10% will not be harvested. Cells will be harvested by scraping with a teflon scraper. The collected cells will be exposed to a hypotonic solution (0.075 M) of potassium chloride at 37 ± 1°C and then fixed using absolute methanol, followed by 3:1 methanol:glacial acetic acid. The fixed cells will be stored in a -20°C freezer prior to slide preparation.

10.8.2 **Slide Preparation and Staining:** To prepare slides, the cells will be collected by centrifugation and resuspended in fresh fixative. Several drops of fixed cells will be dropped onto a slide, air-dried, and stained with Giemsa.

10.8.3 **Scoring:** Slides will be scored without knowledge of the dose group. In addition to the negative, positive, and nitrogen controls, the three highest treatment doses capable of providing a suitable number of metaphase cells for analysis will be scored for clastogenic damage. Selection of which doses to score will be made by the study director. For chromosomal aberration analysis, metaphase cells with 21 ± 2 centromeres will be examined at 1000x magnification. Whenever possible, a minimum of 200 metaphase spreads from each dose group (100 per duplicate culture) will be scored for chromatid and chromosome gaps and breaks, chromatid and chromosome rearrangements, pulverized chromosome(s), pulverized cells, and severely damaged cells (>10 aberrations)(5). If less than 100 cells are obtained from any culture, the balance of the cells, to the extent possible, will be scored from the duplicate culture. The number and types of aberrations found will be presented for each treatment group. The percentage of structurally damaged cells in the total population of cells examined will be calculated for each group. The frequency of structural aberrations per cell will also be calculated and reported for each group.

In addition, for each culture, the proportion of cells at metaphase (mitotic index), based on 1000 cells per culture, and the polyploid index, based on 100 metaphase cells per culture, will be assessed. In assessing the polyploid index, the proportion of endoreduplicated mitosis will be determined.

10.9 **Statistical Analysis:** A decision to classify a chromosomal aberration response as negative, equivocal, or positive must involve a consideration of the appropriateness of the concurrent control data, a formal statistical analysis of the experimental data, and interpretation as to the biological relevance of the response by an experienced scientific

investigator. An alpha level of 0.05 will be used to indicate statistical significance in all analyses. The percentage of damaged cells and the frequency of aberrations per cell will be determined for each culture and dose. The statistical analysis of the percentage of metaphase cells containing at least one chromosomal aberration (excluding gaps) will involve a one-tailed Cochran-Armitage trend test and a one-tailed pairwise Fisher's exact test comparison of each treatment group against the concurrent negative control treatment group (6). The mitotic index and the polyploid index data will be statistically analyzed by analysis of variance and a two-tailed pairwise comparison of each treatment group against the concurrent negative control treatment group.

11.0 Criteria for Determination of a Valid Test

11.1 Negative Control: The mean percentage of cells with structural aberrations in the negative control must not exceed 6%.

11.2 Positive Control: The positive control set of cultures will exhibit a statistically significant increase in the percentage of metaphase cells with at least one chromosomal aberration (excluding gaps).

11.3 Test Substance: The test substance, at least at the highest dose, should induce at least a 50% depression in the mitotic index. However, if no cytotoxicity is observed at the limit of exposure, the assay will be considered acceptable.

12.0 Criteria for a Positive Response

The response to the test substance will be deemed positive if the following criteria are met:

A significant, dose-dependent increase in the percentage of metaphase cells containing at least one chromosomal aberration (excluding gaps) is detected. This is demonstrated by a statistically significant finding in the one-tailed trend test.

A statistically significant increase for at least one treatment dose is detected in the percentage of metaphase cells containing at least one chromosomal aberration (excluding gaps), as indicated by an appropriate pairwise comparison of each dose against the concurrent negative control treatment group.

If either, but not both, of the above conditions are met, the assay results will be evaluated by the study director and be classified as positive, equivocal, or negative depending on the nature and magnitude of the response.

If neither of the above conditions are met, the test substance is classified as negative for clastogenic activity in this *in vitro* test.

13.0 Records to be Maintained:

Data will be recorded on loose work sheets adapted or prepared as necessary for the test results. All data, slides, original copies of the protocol and report, and all correspondence will be archived at ILS until acceptance of the final report by the sponsor or three months after submission of the final report to the sponsor. At this time all items will be transferred to the sponsor for archiving.

14.0 Report:

A final report will be provided to the sponsor upon completion of the study. The report will contain a summary of results, experimental design, materials, test procedures, and results. Data are presented in tabular form for the relative toxic effects of treatment, the number and types of aberrations per cell, and the frequency of cells with aberrations. The report will be audited by the ILS Quality Assurance Unit (QAU). An appropriate QA statement will be added to the report at the time of final issuance.

15.0 Good Laboratory Practices Compliance:

The study will be conducted in accordance with Good Laboratory Practice regulations as promulgated by the U.S. Environmental Protection Agency (40 CFR Part 792). The protocol will be reviewed by the ILS Quality Assurance Unit before final approval. A quality assurance inspection of critical phases will be conducted to assure the quality and integrity of the study results. An audit of the final report will be conducted to determine the consistency between the reported information and the raw data.

16.0 Personnel Health and Safety:

Safety procedures will be adhered to as stated in the ILS Health and Safety Manual and standard operating procedures. At the termination of each experiment, all waste materials will be disposed of in those areas specifically designed for the disposal of hazardous substances.

17.0 Test Substance Disposition:

Any unused test substance and a log accounting for all test substance use will be returned to the sponsor upon completion of the contract.

18.0 References:

1. Preston, R.J., Au, W., Bender, M.A., Brewen, J.G., Carrano, A.V., Heddle, J.A., McFee, A.F., Wolff, S., and Wassom, J.S. (1981) Mammalian *in vivo* and *in vitro* cytogenetic assays: A report of the Gene-Tox Program. Mutat. Res. 87: 143-188.

2. Scott, D., Galloway, S.M., Marshall, R.R., Ishidate, M., Brusick, D., Ashby, J., and Myhr, B. (1991) Genotoxicity under extreme culture conditions. A report from ICPEMC Task Group 9. Mutation Res. 257: 147-204.

3. Bean, C.L., Armstrong, M.J., and Galloway, S.M. (1992) Effect of sampling time on chromosome aberration yield for 7 chemicals in Chinese hamster ovary cells. Mutat. Res. 265: 31-44.

4. Kram, D., Bynum, G.D., and Tice, R.R. (1981) Bromodeoxyuridine labelling of mammalian chromosomes for the analysis of sister chromatid exchanges and cell replication kinetics. In: "Current Trends in Morphological Techniques". J.E. Johnson (ed.), CRC Press, Inc., Boca Raton, FL, pp. 147-176.

5. Savage, R.K.J. (1975) Classification and relationships of induced chromosomal structural changes. J. Med. Genetics, 12: 103-122.

6. Margolin, B.H., Resnick, M.A., Rimpo, J.Y., Archer, P., Galloway, S.M., Bloom, A.D., and Zeiger, E. (1986) Statistical analysis for *in vitro* cytogenetic assays using Chinese Hamster Ovary cells. Environmental Mutagenesis 8: 183-204.

19.0 Approvals:

Sponsor (Project Officer):_____ Date:_____

Study Director: Raymond Tice Date: March 12, 1996

2. Scott, D., Galloway, S.M., Marshall, R.R., Ishidate, M., Brusick, D., Ashby, J., and Myhr, B. (1991) Genotoxicity under extreme culture conditions. A report from ICPEMC Task Group 9. Mutation Res. 257: 147-204.

3. Bean, C.L., Armstrong, M.J., and Galloway, S.M. (1992) Effect of sampling time on chromosome aberration yield for 7 chemicals in Chinese hamster ovary cells. Mutat. Res. 265: 31-44.

4. Kram, D., Bynum, G.D., and Tice, R.R. (1981) Bromodeoxyuridine labelling of mammalian chromosomes for the analysis of sister chromatid exchanges and cell replication kinetics. In: "Current Trends in Morphological Techniques". J.E. Johnson (ed.), CRC Press, Inc., Boca Raton, FL, pp. 147-176.

5. Savage, R.K.J. (1975) Classification and relationships of induced chromosomal structural changes. J. Med. Genetics, 12: 103-122.

6. Margolin, B.H., Resnick, M.A., Rimpo, J.Y., Archer, P., Galloway, S.M., Bloom, A.D., and Zeiger, E. (1986) Statistical analysis for *in vitro* cytogenetic assays using Chinese Hamster Ovary cells. Environmental Mutagenesis 8: 183-204.

19.0 Approvals:

Sponsor (Project Officer): Lt Roy W. Mather Date: ~~March 14~~, 1996 May 23 May 96

Study Director: Raymond Tice Date: March 12, 1996

The undersigned reviewed and approved this protocol in February 1996 but neglected to sign it at that time.

Lt Roy W. Mather
23 May 96

INTEGRATED LABORATORY SYSTEMS

PROTOCOL AMENDMENT FORM

Sponsor Study Number: DAAD05-91-C-0018 Protocol Amendment Number: 1

ILS Project Number: A073-004 ILS Chemical Repository # 96-01

Sponsor Code: A1

Study Title: In Vitro Chromosome Aberrations Study in Chinese Hamster Ovary (CHO) Cells

Amendment Made: The toxicity test in the presence of metabolic activation will be repeated over the same test article dose range.

Section Amended: 10.7 Toxicity Assay

Reason for Amendment: The toxicity test was deemed unacceptable due to the lack of mitosis combined with the high osmolality in the control cultures

Raymond Tice 4/9/96

Study Director Date

_____ /

Sponsor (if applicable) Date

INTEGRATED LABORATORY SYSTEMS

PROTOCOL AMENDMENT FORM

Sponsor Study Number: DAAD05-91-C-0018 Protocol Amendment Number: 2

ILS Project Number: A073-004 ILS Chemical Repository # 96-01

Sponsor Code: A1

Study Title: In Vitro Chromosome Aberrations Study in Chinese Hamster Ovary (CHO) Cells

Amendment Made: The doses to test for clastogenicity in the absence of metabolic activation will be 100% air, 50%, 60%, 70%, 80%, 90%, 100% FE-13 and 100% nitrogen. The exposure will be for 4 hours, and the duration between the onset of exposure and culture termination will be 19 hours.

Section amended: 10.4 Dose Levels; 10.8 Chromosomal Aberration Assay

Reason for amendment: Selection of 100% as the highest dose to test in the absence of metabolic activation is based on the results of the toxicity test which indicated that this doses should induce at least a 50% depression in mitotic index. Selection of 19 hours as the duration between the onset of exposure and culture termination is based on obtaining a time which will be approximately 1.5 times the duration of the normal cell cycle. In the -S9 toxicity test, the average generation time for the control cultures was calculated to be 12.57 hours; a 19-hour incubation period is equal to 1.51 cell cycle durations.

Raymond Tice, 4/9/96

Study Director Date

Sponsor (if applicable) Date

INTEGRATED LABORATORY SYSTEMS

PROTOCOL AMENDMENT FORM

Sponsor Study Number: DAAD05-91-C-0018 Protocol Amendment Number: 3

ILS Project Number: A073-004 ILS Chemical Repository # 96-01

Sponsor Code: A1

Study Title: In Vitro Chromosome Aberrations Study in Chinese Hamster Ovary (CHO) Cells

Amendment Made: The doses to test for clastogenicity in the presence of metabolic activation will be 100% air, 50%, 60%, 70%, 80%, 90%, 100% FE-13 and 100% nitrogen. The exposure will be for 4 hours, and the duration between the onset of exposure and culture termination will be 19 hours.

Section amended: 10.4 Dose Levels; 10.8 Chromosomal Aberration Assay

Reason for amendment: Selection of 100% as the highest dose to test in the presence of metabolic activation is based on the results of the toxicity test which indicated that this dose is the highest dose possible for this test article and that a 50% depression in mitotic index was not observed at this dose. Selection of 19 hours as the duration between the onset of exposure and culture termination is based on obtaining a time which will be approximately 1.5 times the duration of the normal cell cycle. In the +S9 toxicity test, the average generation time for the control cultures was calculated to be 12.54 hours; a 19-hour incubation period is equal to 1.52 cell cycle durations.

Raymond Tice 4/24/96

Study Director Date

/

Sponsor (if applicable) Date

INTEGRATED LABORATORY SYSTEMS

PROTOCOL DEVIATION

SPONSOR CODE: A1 PROTOCOL DEVIATION #: 1

ILS PROJECT NO.: A073-004 CHEMICAL REPOSITORY #: 96-01

STUDY TITLE: In Vitro Chromosome Aberrations Study in CHO Cells

Deviation:

The FE-13 tank was stored in the treatment lab rather than the chemical repository for the duration of the study.

Cause of the Deviation:

Due to the extensive rotometer connections necessary for dosing a gas, the tank was left in the treatment lab for logistical reasons, chained to a tank carrier.

Corrective Action Taken:

None

Impact on Study:

None

Submitted by:

Raymond Tice
Study Director

5/22/96
Date

INTEGRATED LABORATORY SYSTEMS

PROTOCOL DEVIATION

SPONSOR CODE: A1 PROTOCOL DEVIATION #: 2

ILS PROJECT NO.: A073-004 CHEMICAL REPOSITORY #: 96-01

STUDY TITLE: In Vitro Chromosome Aberrations Study in CHO Cells

Deviation:

The Study Protocol was not signed by the Project Officer prior to starting the study.

Cause of the Deviation:

Although the Study Protocol was reviewed by the Project Officer prior to starting the study and his approval was provided verbally, he inadvertently did not sign the protocol until after the study was started and we did not document his approval.

Corrective Action Taken:

None

Impact on Study:

None

Submitted by:

Raymond Tice 5/24/96

Study Director Date

USACHPPM

U.S. Army Center for Health Promotion and Preventive Medicine

Mutagenicity Testing of FE-13

Readiness Thru Health

Executive Summary

Since the fire extinguishant, Halon 1301 poses an atmospheric ozone depletion potential and environmental regulations no longer allow its production, a suitable replacement must be found. One product currently under examination as a replacement for Halon 1301 is FE-13. (Freon 23; trifluromethane; CHF_3) is a halogenated hydrocarbon considered to be chemically inert although it can release fluoride when exposed to flame or red-hot metal. The median lethal concentration (LC_{50}) of FE-13, based on a 4-hour exposure, is >650,000 ppm. A Toxicity Profile developed for The Army Program Executive Office, Armored Systems Modernization by the Toxicology Division, AEHA in 1994 indicated that no effects were observed for FE-13 in 90-day exposure regimes at 10,000 ppm (1%). The effective extinguishant concentration of FE-13 is 12%. The no observable adverse effects level (NOAEL), based on cardiac sensitization, for FE-13 is greater than 30%. The Toxicity Profile indicated that further testing was necessary to determine developmental, reproductive and mutagenicity potential.

Genotoxic testing is an important component of a toxicological profile. Compounds which induce alterations in nucleic acids and associated components are considered to be genotoxic. Mutagenic testing is a specific type of genotoxic testing. Mutagens can induce types of stable changes in the nucleotide sequence of genes, the chromosome structure, or the chromosome number. These types of genetic events are responsible for a large proportion of human genetic diseases and congenital defects.

The compound FE-13 was tested for its mutagenic potential using four separate test systems. Each test system examined a specific mutagenic component. These test procedures included both *in vivo* and *in vitro* assays.

The AS52/GPT mammalian mutagenesis assay examines a compound's ability to induce gene mutations in the genes which code for the enzyme guanine phosphoribosyl-transferase (*gpt*) of cultured AS52 Chinese hamster ovary cells[1]. The addition of the metabolic activator, S9, allows the identification of promutagens. This test procedure is capable of identifying agents which cause small and large deletion mutations as well as point mutations. Also, this assay can demonstrate the cytotoxicity of the compound by comparing the cloning efficiency of treated cultures with that of nontreated cultures. Cultures were exposed to air concentrations of FE-13 for five hours at 37°C. Concentrations of FE-13, with and without the S9 activator, were 10, 25, 50, 75, and 100%. Some cultures were exposed to 100% nitrogen in order to determine the effects of oxygen deprivation. FE-13 did not induce a significant level of mutagenicity in the presence or absence of metabolic activation.

The *Salmonella typhimurium* / microsome reverse mutation assay (Ames test), developed by Bruce Ames, is an elegant assay for the determination of mutagenicity[2]. This assay employs bacterial strains that are unable to manufacture histadine and is capable of detecting both base pair substitutions and frameshift mutations. The metabolic activator, S9, is used in this test to identify promutagens. The concentrations of FE-13 used in this test procedure were 10, 50, and 100% per plate. FE-13 did not induce a significant level of mutagenicity in the presence or absence of metabolic activation.

In vitro Chromosome aberrations can also be examined using Chinese hamster ovary (CHO) cells[1]. This assay is sensitive to clastogenic activity of a variety of chemicals. The

detection of a significantly elevated level of chromosome damage is considered an indicator of genetic damage. The S9 fraction of rat liver homogenate is also used in this test system to identify promutagens. Toxicity of FE-13 was examined in cultures, with and without S9, using concentrations of 5, 10, 25, 50, 75, and 100% and a four hour exposure period. One group was exposed to 100% nitrogen in order to determine the effects of oxygen deprivation. This procedure examined average generation time, mitotic index, polyploid index, and cell density. Clastogenic activity was evaluated using concentrations of 50, 60, 70, 80, 90, and 100% in the presence or absence of the S9 activator. As with the toxicity portion of this study, one group was exposed to 100% nitrogen in order to determine the effects of oxygen deprivation. A continuous exposure protocol in the absence of S9 was not practical due to the potential adverse effects of oxygen deprivation. FE-13 was found to induce a significant level of clastogenic damage in concentrations of 80% and above in the absence of metabolic activation. Control (air only) cultures containing S9 displayed a 2% (not statistically significant) increase in cellular damage while nonactivated cultures displayed no damage. This difference in baseline activity may have accounted for the nonsignificant increase in cellular damage with the S9 activator although the level of damage from exposure was identical with and without the S9 activator. Cells exposed to 100% nitrogen also displayed the same level of damage. Damage, therefore, is probably due to a decreased oxygen level rather than the activity of FE-13.

The mouse bone micronucleus assay is an *in vivo* test system which can determine the ability of a compound to induce micronuclei formation in immature erythrocytes of male and female mice[3]. Micronuclei are formed when chromosomes lag or fragment during cell division. The B6C3F1 strain of mouse was used in this study as this strain appears to be exquisitely sensitive to micronucleus induction. This assay is the most reliable method for evaluating the potential of a compound to induce clastogenic or aneugenic damage. FE-13 was assayed using concentrations of 13%, 26%, 50%. Control animals were exposed to 100% air as well as an oxygen poor environment of 50% air and 50% nitrogen. FE-13 did not induce a significant level of mutagenicity.

The results of the above tests indicate that FE-13 does not induce a mutagenic effect at dosage levels tested and, from a mutagenicity standpoint, it appears to be a suitable replacement for Halon 1301. Further genotoxicity testing of this material is not indicated at this time.

References

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